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MicroRNA-10a Binds the 5′UTR of Ribosomal Protein mRNAs and Enhances Their Translation

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SUMMARY

MicroRNAs (miRNAs) are small RNAs that function as posttranscriptional regulators of gene expression. miRNAs affect a variety of signaling pathways, and impaired miRNA regulation may contribute to the development of cancer and other diseases. Here we show that miRNA miR-10a interacts with the 5′ untranslated region of mRNAs encoding ribosomal proteins to enhance their translation. miR-10a alleviates translational repression of the ribosomal protein mRNAs during amino acid starvation and is required for their translational induction following anisomycin treatment or overexpression of RAS. We show that miR-10a binds immediately downstream of the regulatory 5′TOP motif and that the 5′TOP regulatory complex and miR-10a are functionally interconnected. The results show that miR-10a may positively control global protein synthesis via the stimulation of ribosomal protein mRNA translation and ribosome biogenesis and hereby affect the ability of cells to undergo transformation.

INTRODUCTION

miRNAs constitute a class of short, noncoding RNAs that posttranscriptionally regulate gene expression in multicellular organisms by interaction with partially complementary target sites in mRNAs (Pillai et al., 2007). miRNAs recognize their target sites by incomplete base pairing, and individual miRNAs may regulate a cohort of mRNAs. Consequently, miRNAs have been found to affect a multitude of signaling pathways (Pillai et al., 2007). Human miRNA genes are frequently located in cancer-associated genomic regions, and perturbed miRNA expression patterns have been observed in many human cancers (Calin et al., 2005; He et al., 2005; Johnson et al., 2005). In addition, a number of studies have demonstrated causal links between miRNA deregulation and cancer-related processes (Costinean et al., 2006; He et al., 2005; Voorhoeve et al., 2006).

Targets of miRNA regulation are inherently difficult to identify due to the partial complementarity between the miRNAs and the target mRNA. Focus has largely been on computational predictions of targets based on the observation that many miRNAs can recognize their targets by binding to motifs in the 3′ untranslated region (UTR) sequences complementary to bases 2–8 of the miRNA (the seed region). The challenge of establishing miRNA functions and understanding the biological processes they regulate has emphasized the need for new experimental approaches to identify miRNA targets. In the present study, we use a direct affinity-based procedure to isolate mRNA targets bound by miR-10a and identify ribosomal protein (RP) mRNAs as functionally important targets for miR-10a.

The miRNAs miR-10a and miR-10b are close homologs, differing by a single central nucleotide only. In the mouse embryo, miR-10a is mainly expressed in a region of the posterior trunk (Mansfield et al., 2004), whereas miR-10a in adult mice is broadly expressed with the highest levels found in kidney, muscle, lung, and liver (Beuvink et al., 2007; Landgraf et al., 2007). The miR-10a homolog miR-10b is highly overexpressed in several tumor types and is reportedly involved in the progression of cancer (Garzon et al., 2006; Ma et al., 2007).

The translational machinery is tightly regulated in mammalian cells, and this is in part mediated by the controlled translation of RPs (Ruggero and Pandolfi, 2003). The translation of RPs and other proteins involved in protein synthesis is regulated via a 5′TOP motif rendering the transcripts sensitive to cellular stress signals and nutrient status (Meyuhas, 2000). We show that miRNA miR-10a interacts with the 5′UTR of mRNAs encoding RPs and enhances their translation. miR-10a binds immediately downstream of the regulatory 5′TOP motif, and the 5′TOP is necessary for miR-10a translational enhancement. The results indicate that miR-10a may positively control global protein synthesis via stimulation of RP mRNA translation and that the 5′TOP regulatory complex and miR-10a are functionally interconnected.

RESULTS

miR-10a Associates with Ribosomal Protein mRNAs

To identify mRNA targets for miR-10a, we employed an affinity-based target-identification procedure in which the miRNA of interest is synthesized with a 3′ biotin group allowing for

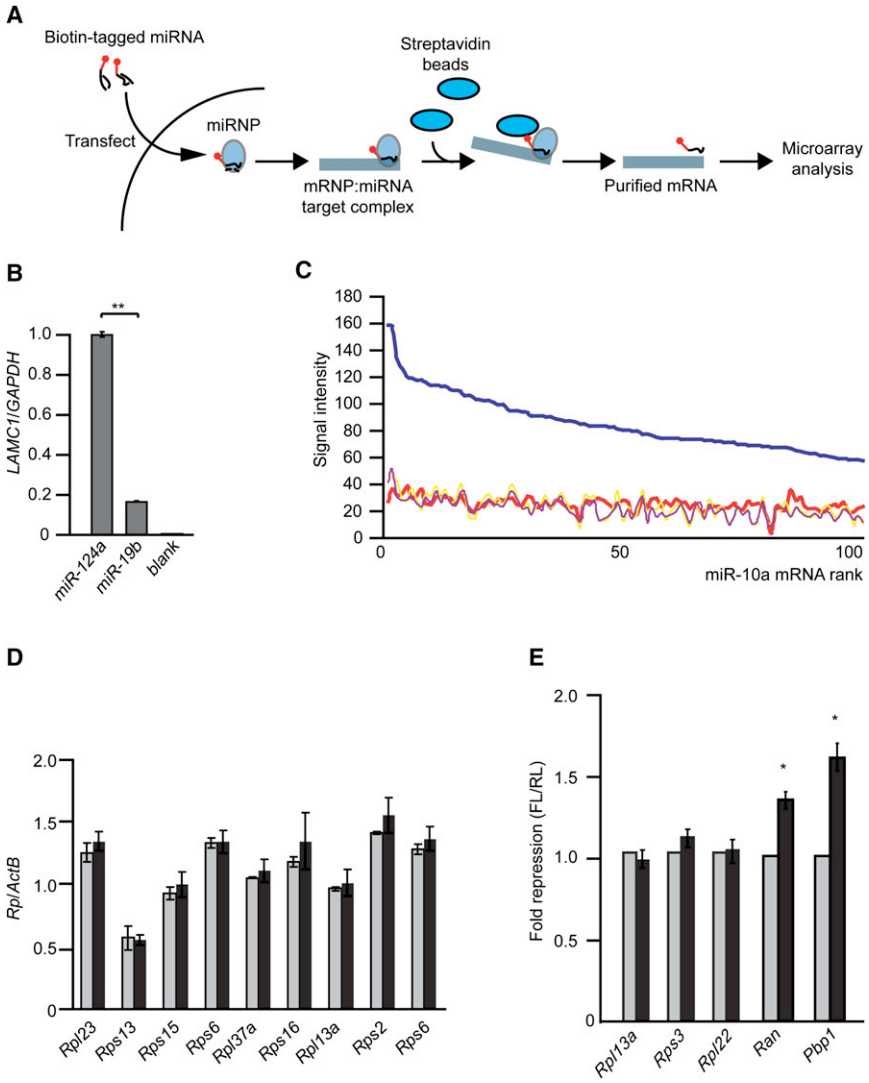

Figure 1. Affinity Purification of miR-10a Targets

(A) Schematic outline of the affinity purification procedure.

(B) Affinity purification with biotin-tagged miR-124a from human neuronal U87 cells and RT-qPCR for its endogenous target *LAMC1* relative to *GAPDH*. **$p < 0.01$, error bars represent SD.

(C) Microarray data from affinity purifications with miR-10a (blue), miR-10a MUT (yellow), let-7c (purple), and blank (red, no miRNA). Data from triplicate experiments for each miRNA were pooled and analyzed on cDNA microarrays. Shown are normalized expression values for each miRNA for the 100 most enriched mRNAs in miR-10a compared to miR-10a MUT from two independent experiments.

(D) Levels of the indicated RP mRNAs relative to *ActB* from triplicate microarray analyses. Grey bars, bantam; black bars, miR-10a.

(E) 3′UTR luciferase reporter assay for the indicated mRNAs. Grey bars, bantam; black bars, miR-10. Y axes show relative values for Firefly luciferase to *Renilla reniformis* luciferase and are normalized to bantam transfected cells. *$p < 0.05$, all experiments performed at least three times.

subsequent purification with streptavidin (Figure 1A). We have previously verified this technique for affinity purification of miRNA targets in *Drosophila melanogaster* cells (Orom and Lund, 2007). We validated the technique for mammalian cells using a biotin-tagged miR-124a targeting *LAMC1* (Cao et al., 2007). Affinity purification experiments in neuronal U87 cells, which express endogenous miR-124a, resulted in a ~5-fold enrichment of the endogenous *LAMC1* target with miR-124a compared to control miR-19b, and >100-fold enrichment compared to mock-transfected cells (Figure 1B). To identify targets of miR-10a, biotin-tagged miR-10a, a mutant control miR-10a containing three substitutions in the 5′ end, and a let-7c control miRNA were transfected into mouse E14 embryonic stem (ES) cells. Western blot analysis confirmed that the biotin-labeled miR-10a incorporated into functionally active Ago2-containing complexes (see Figures S1A and S1B available online). Following cell lysis, miRNA/protein/mRNA complexes were purified on streptavidin-agarose beads and the associated mRNAs isolated and identified by microarray analysis. Results from two independent triplicate affinity purification experiments were analyzed and ranked according to the relative enrichment in the miR-10a affinity purifications after normalization. As shown in Figure 1C, miR-10a copurifies with a specific subset of mRNAs not enriched by any of the control miRNAs. Gene ontology analyses revealed that 55 of the 100 most enriched probe sets represent mRNAs encoding proteins involved in protein biosynthesis and in particular RPs (Figures S2A and S2B and Table S1) (Dennis et al., 2003).

The levels of the RP mRNAs were unaffected upon miR-10a transfection as compared to mock-transfected cells (Figure 1D), indicating that miR-10a mediates predominantly translational regulation of the RP mRNAs. To study the translational regulation, we inserted the 3′UTRs of *Rpl13a*, *Rps3*, *Rpl22*, *Ran*, and *Pbp1* downstream of a luciferase reporter and measured the effect of miR-10a on *luc* expression. Whereas the RP 3′UTRs are short and do not contain miR-10a complementary seed sites, *Ran* and *Pbp1* 3′UTRs contain sites complementary to the seed region of miR-10a. While miR-10a imposed a significant repression on transcripts containing *Ran* and *Pbp1* 3′UTRs, reporters containing RP 3′UTRs were unresponsive to miR-10a (Figure 1E).

miR-10a Enhances Translation of RP mRNAs

To examine the effect of miR-10a on the synthesis of RPs, we immunoprecipitated the RPs Rps16, Rps6, and Rpl9 from ES cells pulse-labeled with [^{35}S]-methionine following transfections with miR-10a, a bantam control miRNA, or a miR-10a inhibitor (Figure 2A). Immunoprecipitation of a nonribosomal protein,

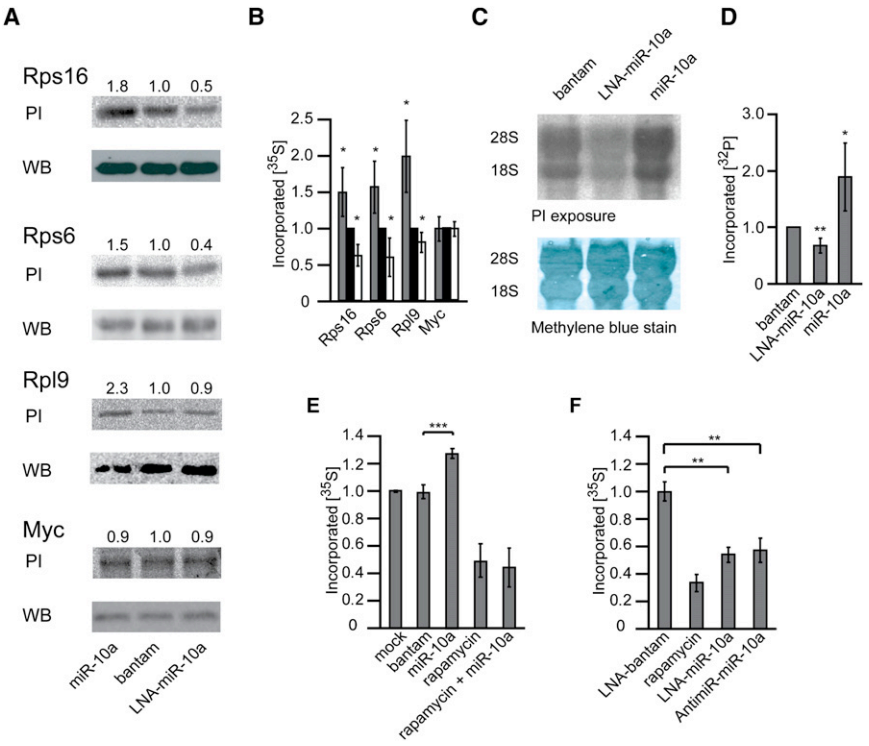

Figure 2. miR-10a Enhances Synthesis of RPs

(A and B) Immunoprecipitations of [^{35}S]-pulse-labeled cells 24 hr after transfections with miR-10a, a bantam control, or a miR-10a inhibitor with antibodies against the indicated proteins. PI, phosphoimager exposure 96 hr; WB, western blots of the immunoprecipitated proteins as loading control. Rps16 IP was performed four times, and Rps6 and Rpl9 were repeated three times and quantified in (B). Grey bars, miR-10a; black bars, bantam; white bars, LNA-miR-10a. *$p < 0.05$, error bars represent SD.

(C) Ribosome biogenesis measured by [^{32}P] incorporation 24 hr after transfection (upper panel). Methylene blue staining of the membrane is shown for loading control (lower panel).

(D) Quantification of ribosome biogenesis assay from three independent experiments. Data have been normalized to bantam set to 1. *$p < 0.05$, **$p < 0.01$, error bars represent SD.

(E and F) Total protein synthesis was determined 24 hr after transfection by [^{35}S] pulse labeling of cells and TCA precipitation. (E) Transfected with the indicated miRNAs or (F) inhibitors. **$p < 0.01$, ***$p < 0.001$, error bars represent SD of three independent replicates. Data are representative for at least four independent experiments.

Myc, was included as control. Whereas transfections with miR-10a significantly increased the amount of newly synthesized RPs, transfections with a miR-10a inhibitor resulted in a marked decrease in RP synthesis. Quantifications of incorporated radioactivity compared to the total amount of immunoprecipitated RP are depicted in Figure 2B. The formation of ribosomes is a concerted action dependent on the availability of RPs (Averous and Proud, 2006), and we speculated that miR-10a could affect ribosome biogenesis. Quantifications of newly synthesized rRNA from ES cells transfected with miR-10a or controls and labeled with [^{32}P]-orthophosphate clearly show an increase in the rate of ribosome biogenesis following miR-10a transfections (Figures 2C and 2D). These experiments demonstrate that miR-10a has a positive regulatory effect on RP translation and ribosome biogenesis. We subsequently assessed the effect of miR-10a on the rate of total protein synthesis and found that exogenous miR-10a mediated a ~30% increase in overall protein synthesis compared to controls as determined by TCA precipitation of [^{35}S]-labeled proteins (Figure 2E). The effect of the mTOR kinase inhibitor rapamycin was unaffected by exogenous miR-10a, suggesting that mTOR signaling is required for miR-10a-dependent translational enhancement (Figures 2E and 2F). Importantly, inhibiting endogenous miR-10a resulted in a 40% decrease in global protein synthesis (Figure 2F). Based on this evidence, we propose that miR-10a participates in the control of ribosome biogenesis and global protein synthesis via the regulation of RP mRNA translation.

RP mRNAs, and other mRNAs containing a 5′TOP motif, selectively redistribute from active translation in polysomes to inactive ribonucleoprotein (RNP) complexes upon nutrient deprivation, such as amino acid starvation (Meyuhas, 2000). We examined the effect of miR-10a on endogenous *Rpl13a*, *Rps16*, and *Rpl23* in polysome fractionation experiments. Transfections with miR-10a do not shift RP mRNAs toward heavier fractions when cells are maintained in complete medium with 10% FCS (Figure 3, compare panels A and B). During amino acid starvation, however, 5′TOP mRNAs remained associated to polysomes in cells transfected with miR-10a, in contrast to cells transfected with a control bantam miRNA, in which mRNAs redistribute to the RNPs (Figure 3, compare panels C and D). Quantifications of data from three independent experiments are presented in Figure 3E. These data further support that miR-10a positively affects translation of RPs during amino acid starvation. Since the response to amino acid starvation is specific to 5′TOP-containing mRNAs, the data suggest that miR-10a is functionally linked to the 5′TOP motif and led us to examine if miR-10a interacts with the 5′UTR of RP mRNAs.

RP 5′UTRs Are Directly Bound by miR-10a

To establish whether the miR-10a interaction to RP mRNAs is direct, we applied a photo-inducible 4-thiouridine (tU)-based crosslinking approach (Sontheimer, 1994; Wyatt et al., 1992) and substituted the uridine base in either position 6 or 19 of miR-10a with tU bases. The tU is photoactivated by long-wave UV light and forms crosslinks at close range only (Sontheimer, 1994). The activity of the tU-modified miR-10a duplexes on a miR-10a-sensitive reporter was comparable to that of the unmodified miR-10a (Figure S1B). Following transfections with miR-10a, miR-10a(6tU), or miR-10a(19tU), the cells were lysed and the lysates irradiated with 365 nm UV light to induce crosslinking between miR-10a and the physically associated mRNAs. To disrupt protein-mRNA interactions and noncovalent interactions,

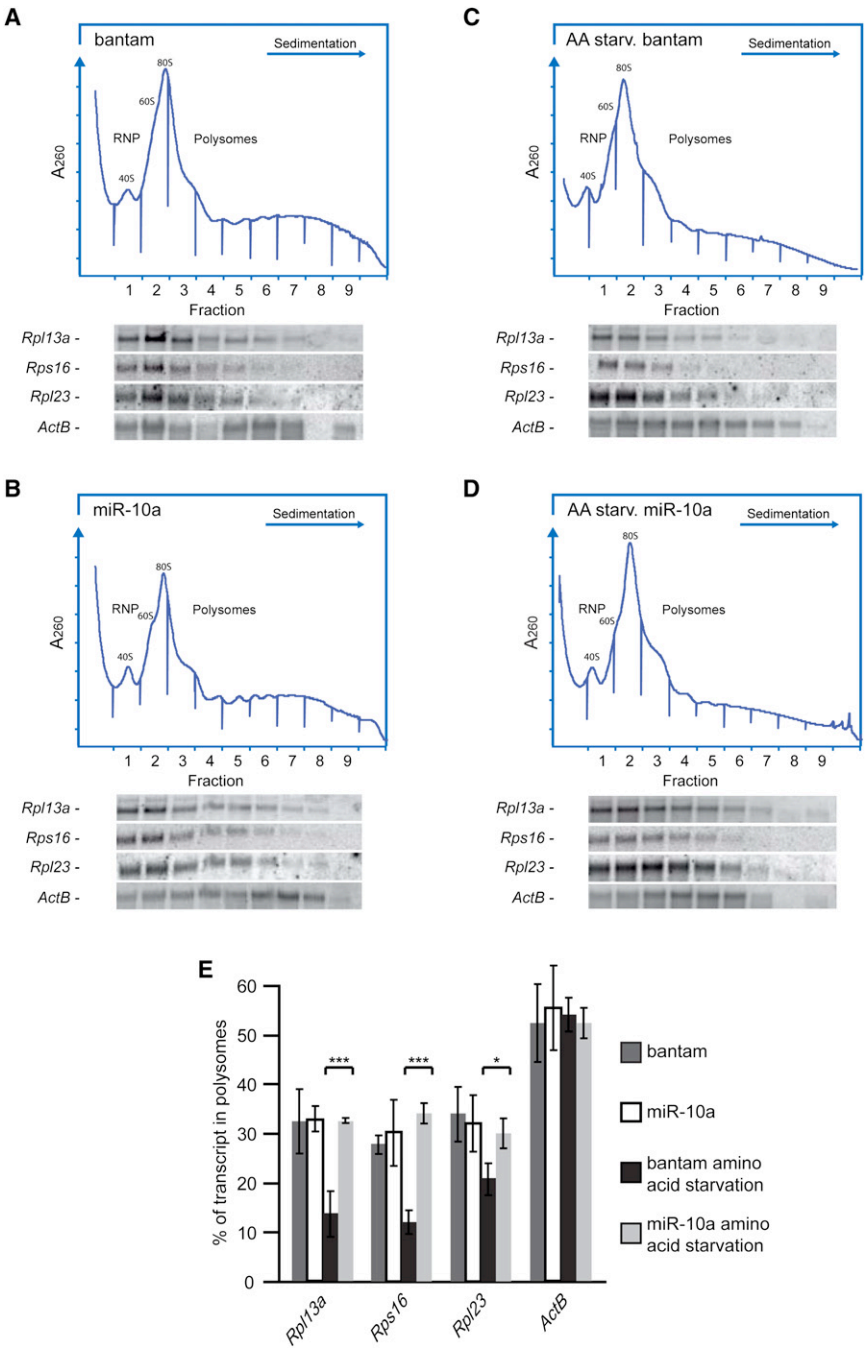

Figure 3. RP Polysome Association during Amino Acid Starvation with miR-10a

(A and B) Polysomal distribution of RP mRNAs in extracts prepared from ES cells transfected with either bantam (A) or miR-10a (B) in complete medium with 10% serum.

(C and D) Polysomal distribution upon amino acid starvation and transfection with (C) bantam or (D) miR-10a. RNA extracted from individual fractions was analyzed with probes specific for endogenous *Rpl13a*, *Rps16*, *Rpl23*, and *ActB*.

(E) Quantification of and statistics on sucrose gradients were done by quantifying the associated mRNA of each fraction as determined by northern blotting for the indicated mRNAs. Relative amount of mRNA in fractions corresponding to ribosome association to total mRNA in all fractions is shown as "percentage in polysomes." Each probe was used on three independent sucrose gradient experiments. ***$p < 0.001$. Error bars represent SD. Sucrose gradients with EDTA were done to control for nonribosomal association of mRNAs with the heavy fractions, and no such association was found (data not shown).

irradiated lysates were purified in TRIzol before the biotin-labeled miRNAs were purified on streptavidin beads along with covalently bound target mRNAs. Using quantitative RT-PCR, we observe an enrichment of the RP mRNAs upon crosslinking only when a tU analog is present in the miRNA, demonstrating that miR-10a binds directly to the RP mRNAs (Figure 4A).

To identify miR-10a-binding sites in the RP mRNAs, we focused on the 5′UTRs and exploited the crosslinking approach using primer extension assays to map the interaction site. Upon UV-induced covalent binding, miR-10a situated at the 5′UTR would constitute a block to the extending cDNA strand, resulting in a product shorter than that for the full-length mRNA (Figure 4B). For these experiments, we used miR-10a(19tU), since this duplex resulted in a higher-affinity purification ratio (Figure 4A). Primer extension experiments on *Rpl13a*, *Rps16*, and *Rps20* from crosslinked total RNA from ES cells result in the appearance of additional bands only in samples transfected with miR-10a(19tU) (Figures 4C–4E). The mapped binding sites are consistent with miR-10a binding the RP 5′UTRs immediately downstream of the 5′TOP motif. We calculated the best interactions between miR-10a and a subset of RP mRNA 5′UTRs using RNAhybrid (Rehmsmeier et al., 2004) (Figure 4F and Figure S3). The calculated best sites of miR-10a recognition are identical to those experimentally mapped for *Rpl13a*, *Rps16*, and *Rps20* and thermodynamically equivalent to, or more stable than, known experimentally validated miRNA target interactions (e.g., miR-155/*TP53INP1* −ΔG = 18.1 kcal/mol and miR-21/*PDCD4* −ΔG = 13.9 kcal/mol) (Frankel et al., 2007; Gironella et al., 2007), supporting the validity of these nonseed miRNA target sites. Hence, in contrast to the majority of experimentally determined miRNA-binding sites, we find miR-10a to bind at the 5′UTR.

5′UTR Requirements for miR-10a-Mediated Translational Upregulation

Since the binding patterns suggested by the mapping data and calculated folding patterns do not involve complete binding of

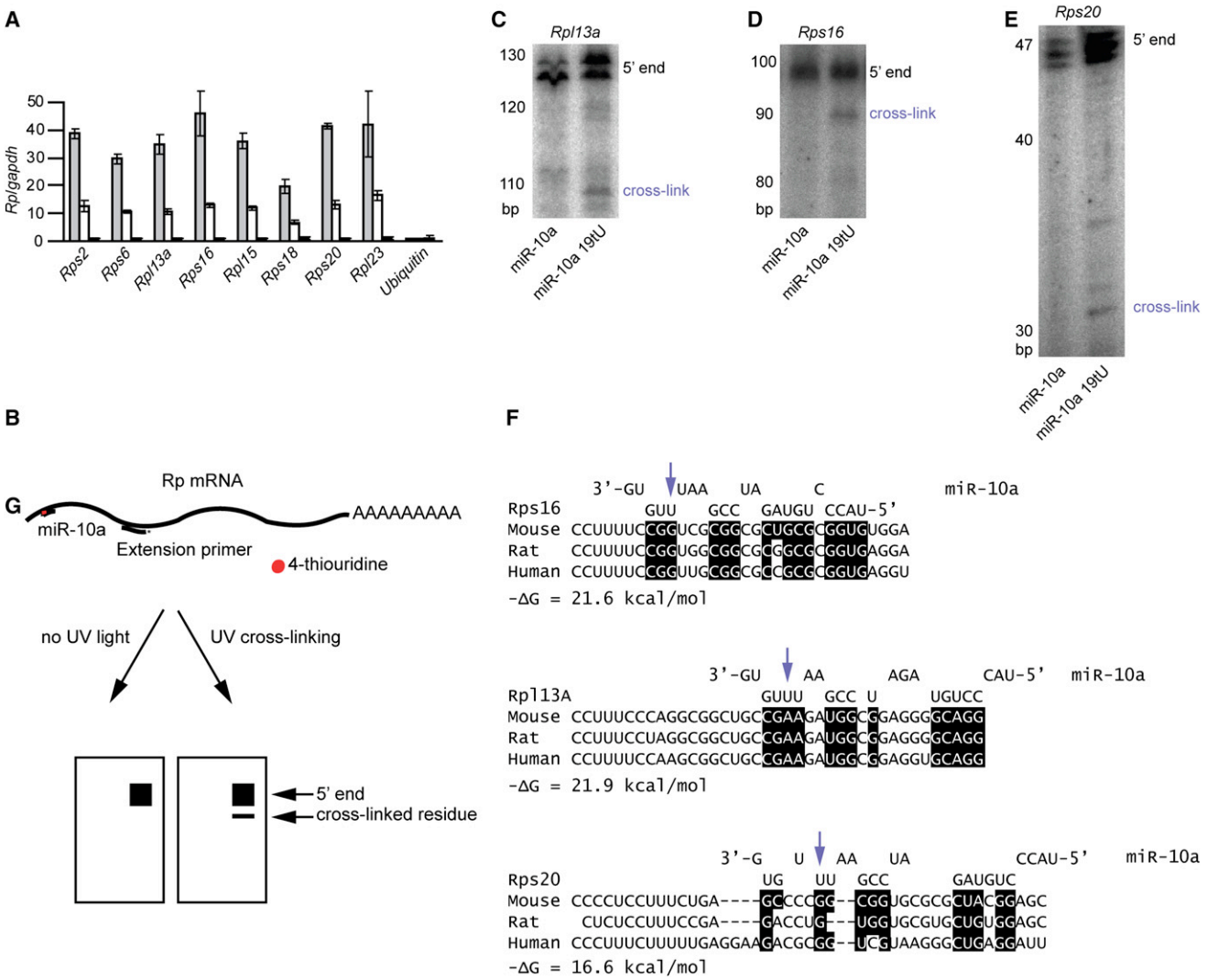

Figure 4. Mapping of miR-10a-Binding Sites

(A) Crosslinked miR-10a associates to RP mRNA. miR-10a with a 4-thiouridine substitution in either position 6 (gray bars), 19 (white bars) from the 5′ end, or no modifications (black bars) were transfected into ES cells that were subsequently exposed to long wave UV light to induce crosslinking of the 4-thiouridine group to bound mRNAs. Shown is RT-qPCR for the indicated RP mRNAs and *Ubiquitin* as a non-RP mRNA control. The y axis shows the indicated mRNA relative to a *Gapdh* specificity control. Data are normalized to the unmodified miR-10a and are representatives of three independent experiments. Error bars represent SD.

(B) Schematic of the crosslinking primer extension approach used to map miR-10a interaction sites in 5′UTRs. The crosslinked miR-10a blocks reverse transcription, causing the appearance of a new band corresponding to the position of miR-10a binding.

(C–E) Primer extension mapping of miR-10a with 4-thiouridine in position 19 to *Rpl13a*, *Rps16*, and *Rps20*. Sequencing gels with the primer extension products were exposed to a phosphoimager screen for 4 days. A longer exposure of the gel shown in (E) did not cause more bands to appear in the control miR-10a lane. Data are representatives of three to six independent replicates.

(F) Thermodynamic calculations for miR-10a binding to 5′UTRs of *Rps16*, *Rpl13a*, and *Rps20*. −ΔG values are calculated with RNAhybrid 2.1. Blue arrows indicate the sites of crosslinking at position 19(tU).

the miR-10a seed region to the 5′UTRs, we validated this interaction further. Toward this, we employed an *Rps16* 5′UTR reporter construct, pS16-WT-Luc, containing a 211 bp region from the *Rps16* gene encompassing the transcriptional start site and 29 nt of exon 1 including the 5′TOP motif and introduced mutations in the 5′TOP motif and in the miR-10a-binding site (Figure 5A). Previous studies have validated that this *Rps16* fragment retains the characteristics of endogenous 5′TOP mRNAs (Levy et al., 1991). Low concentrations of the transcriptional inhibitor anisomycin have previously been found to stimulate translation of 5′TOP mRNAs (Loreni et al., 2000; Nielsen et al., 1995). Accordingly, expression of the pS16-WT-Luc vector was 2.5-fold upregulated in cells treated with 300 nM anisomycin. This induction was abolished when cells were transfected with an inhibitor to miR-10a 24 hr prior to administration of the drug (Figure 5B). The effect is specific to miR-10a inhibition and emphasizes the

requirement for miR-10a during stress-mediated 5′TOP translational regulation. Likewise, translational induction of 5′TOP mRNAs by activated RAS-V12 in NIH 3T3 cells is reduced significantly upon inhibition of miR-10a, pointing to an important role for miR-10a also in response to physiologically relevant stimuli (Figure 5C).

Cotransfection experiments with the various vector mutants and their interaction with miR-10a variants are shown in Figures 5D–5I. Exogenous miR-10a imposes a 60% increase in luciferase activity of the pS16-WT-Luc reporter, compared to bantam or mutated miR-10a controls, thereby providing independent evidence for an enhancing effect of miR-10a on RP translation (Figure 5G). Interestingly, this effect extends to other members of the miR-10 family, suggesting that the positive regulatory effect is conserved among miR-10 family members (Figures S4A and S4B).

To abrogate the miR-10a-binding site, we generated the pS16-comp-Luc vector by mutating 4 nt downstream from the 5′TOP motif of pS16-WT-Luc, corresponding to the positions binding to miR-10a nucleotides 3, 4, 7, and 10 (Figure 5A). These mutations render pS16-comp-Luc inert to miR-10a (Figure 5H). By insertion of compensatory mutations in miR-10a (generating miR-10a comp), we can restore enhancement of translation of the reporter so that miR-10a comp mediates the same positive regulation on pS16-comp-Luc as does miR-10a on pS16-WT-Luc (Figures 5G and 5H). In accordance with the data obtained by primer extension mapping, these experiments independently demonstrate the binding pattern of miR-10a to the 5′UTR of *Rps16*.

To examine the interplay between the 5′TOP motif and miR-10a binding, we substituted five pyrimidines within the 5′TOP motif, thereby creating pS16-CM5-Luc (Figure 5A). Similar mutations have previously been demonstrated to impede 5′TOP properties (Biberman and Meyuhas, 1999). Cotransfections with miR-10a, miR-10a comp, or controls did not affect the translation of the pS16-CM5-Luc reporter, indicating a functional role of the 5′TOP motif in miR-10a-mediated upregulation of RP mRNA translation (Figure 5I). Furthermore, affinity purification of the pS16-CM5-Luc reporter was significantly less efficient than of the pS16-WT-Luc reporter, further supporting a functional interplay between the 5′TOP motif and miR-10a (Figure 5J).

miR-10a Levels Affect Oncogenic Transformation

Several studies have demonstrated that increased translation and deregulated translational control mechanisms are hallmarks of cancer (Averous and Proud, 2006; Gazda et al., 2006; Takagi et al., 2005). We speculated that altered levels of miR-10a could influence the oncogenic properties of transformed cells. NIH 3T3 mouse fibroblasts were transformed with activated RAS-V12 to facilitate anchorage-independent growth and colony formation in soft agar (Figure 6A). To examine the effect of miR-10a, we transfected NIH 3T3/RAS-V12 cells with miR-10a, a miR-10a inhibitor, or controls; seeded the cells in soft agar; and quantified the number of outgrowing colonies after 3 weeks. Whereas exogenous miR-10a mediated an ~2-fold increase in the number of colonies, inhibition of the endogenous miR-10a significantly reduced the number of colonies forming in soft agar (Figures 6A–6D). Correspondingly, we observed an increase in total protein synthesis in NIH 3T3/RAS-V12 cells following miR-10a transfection and global translational repression in NIH 3T3/RAS-V12 transfected with a miR-10a inhibitor (Figure 6E), supporting a model in which increased global translation of proteins increases the oncogenic potential of transforming cells.

The homolog of miR-10a, miR-10b, has been suggested to enhance tumor cell migration and invasion of metastatic breast cancer cells by repressing translation of *HoxD10* (Ma et al., 2007). To determine the possible influence of a miR-10a/*HoxD10* interaction in our assays, we analyzed the cell lines used in this study for *HoxD10* expression. Neither ES cells nor the NIH 3T3/RAS-V12 cells, used for the protein translation and soft agar assays, express noticeable amounts of *HoxD10* as estimated by RT-qPCR (Figure S5A) and western blotting (data not shown). Furthermore, we did not observe any correlation between miR-10a and *HoxD10* levels (Figure S5B). Taken together, the results suggest a *HOXD10*-independent mechanism likely involving miR-10a-mediated upregulation of translation.

DISCUSSION

Experimental Identification of miRNA Targets

Bioinformatics predictions, based primarily on conserved interactions involving the miRNA seed region, have indicated that miRNAs may bind and regulate the translation of a large number of mRNAs (Brennecke et al., 2005; Grimson et al., 2007; Lewis et al., 2005). Whereas these algorithms have been instrumental in many studies of individual miRNA:mRNA interactions, unbiased approaches to study miRNA target recognition are important to discover new features of miRNAs. Experimental approaches to miRNA target prediction have mainly focused on expression array analysis of cells in which individual miRNAs have been overexpressed (Lim et al., 2005) or endogenous miRNAs have been inhibited following transfections with miRNA-inhibitory oligonucleotides (Frankel et al., 2007; Krutzfeldt et al., 2005). These approaches can clearly identify miRNA targets subjected to mRNA degradation upon miRNA binding but are inherently incapable of finding targets regulated primarily at the level of translation. It is currently unknown what proportion of miRNA targets rely exclusively on translational repression and if mechanistic differences exist between miRNAs. Purification and identification of mRNAs directly bound by tagged miRNAs constitutes a strong and unbiased tool for the integration of miRNA functions into known cellular pathways. This method may furthermore enhance our knowledge on miRNA:mRNA interactions with respect to binding motifs, location of binding sites, and the presence of additional signals in the target mRNAs of importance for miRNA recognition.

miR-10a Binds the 5′UTR of Ribosomal Protein mRNAs

We focused our studies on miR-10a, as this miRNA is highly conserved through evolution with respect to both primary sequence and gene localization within the Hox clusters of developmental regulators (Tanzer et al., 2005). miR-10a is moderately expressed in mouse ES cells and has previously been found also in $CD34^+$ hematopoietic progenitor cells (Garzon et al., 2006). Reporter studies in mouse embryos show that miR-10a is predominantly expressed in the posterior trunk of the developing mouse embryo (Mansfield et al., 2004), whereas in the adult

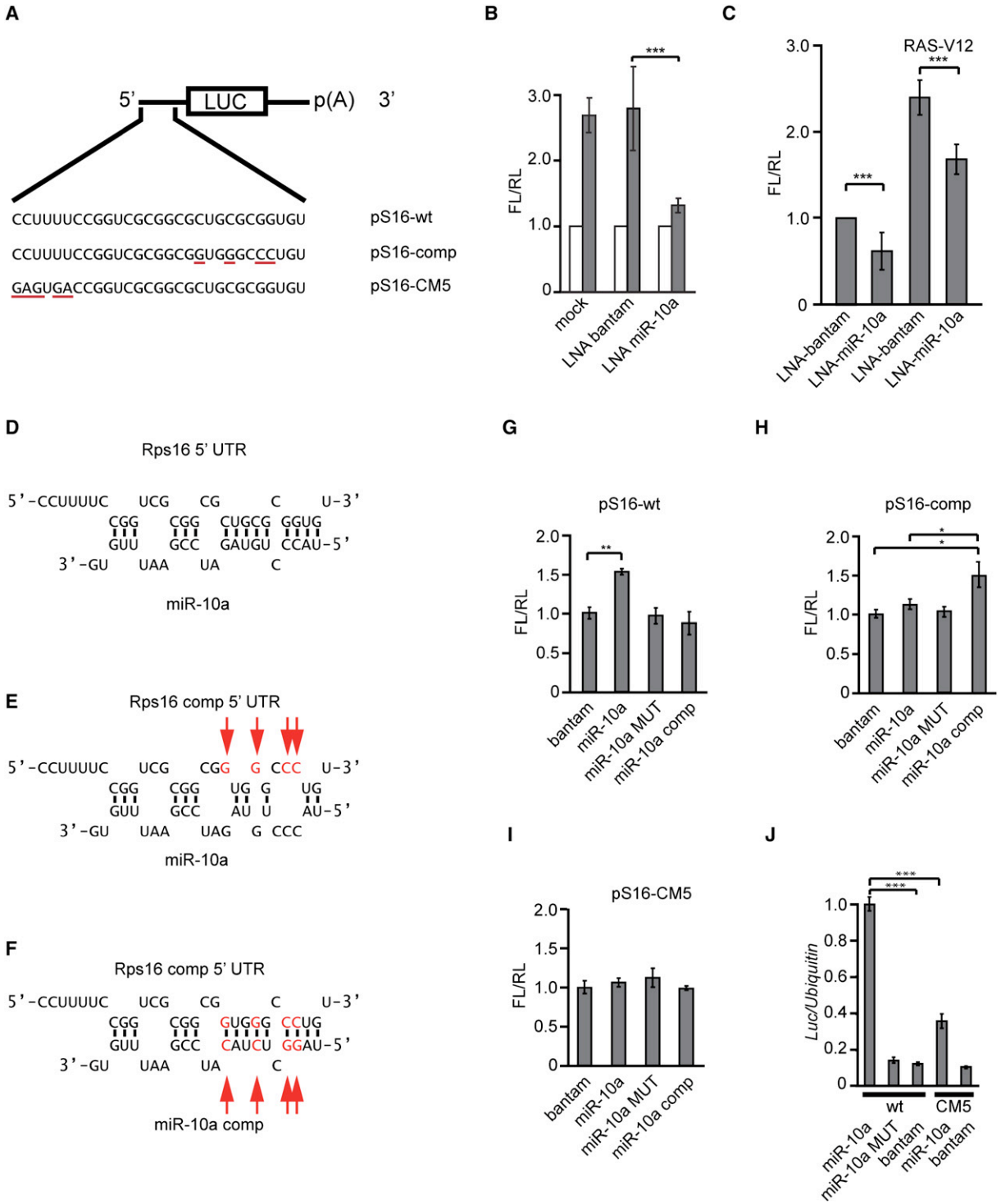

Figure 5. Characterization of the miR-10a Binding to Rps16 5′UTR

(A) Overview of the 5′UTRs of the reporters used.

(B) Anisomycin induces translation of 5′TOP mRNAs when administered in low concentrations. Cells were transfected with the pS16-WT construct along with a control plasmid encoding *Renilla reniformis luciferase* and the indicated miRNA inhibitors. White bars, untreated; gray bars, anisomycin treated. The y axis shows ratio of the Firefly luciferase to control *Renilla reniformis* luciferase. ***$p < 0.001$, error bars represent SEM from three independent experiments.

(C) Translational 5′TOP induction by activated RAS in NIH 3T3 cells. Cells were transfected with pS16-WT construct along with a *Renilla reniformis luciferase* vector and the indicated miRNA inhibitors. Data are representative of three independent experiments. ***$p < 0.001$, error bars represent SD.

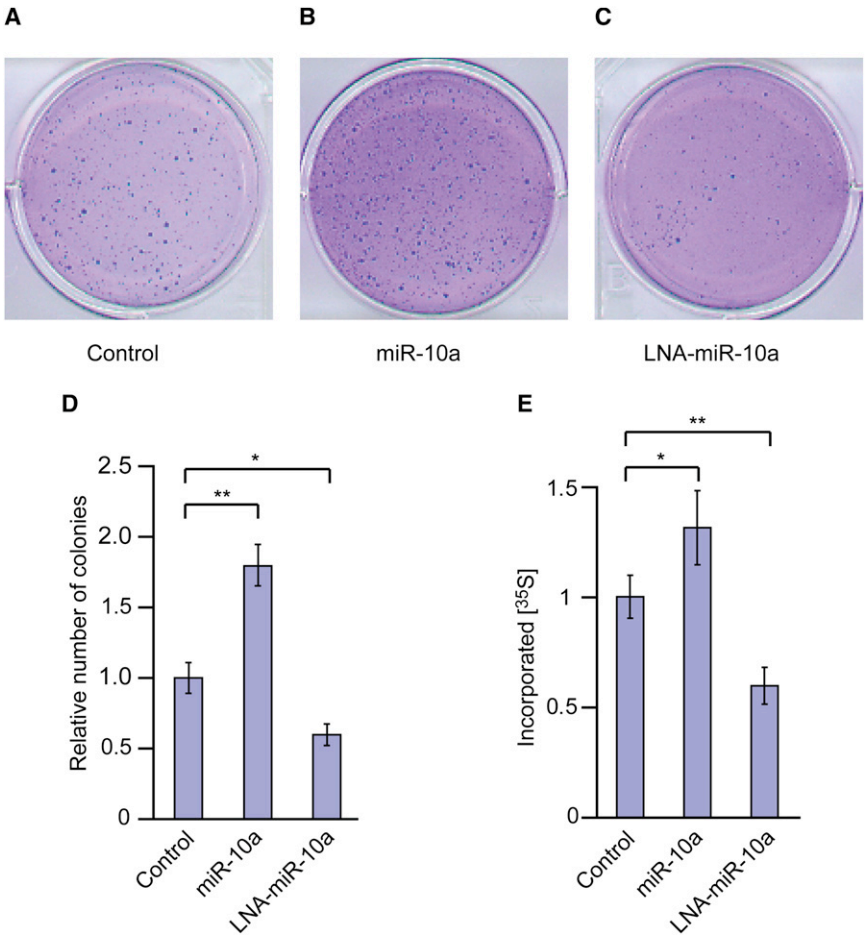

Figure 6. miR-10a Induces Oncogenic Transformation

Mouse NIH 3T3 cells were transduced with oncogenic RAS-V12 and subsequently transfected on two consecutive days with either (A) bantam control miRNA, (B) miR-10a, or (C) a miR-10a inhibitor and seeded in soft agar. The number of colonies was quantified after 2 weeks. *$p < 0.05$, **$p < 0.01$, error bars represent SEM of four independent experiments. Quantifications of colonies are normalized to the bantam-transfected control cells and are shown in (D). (E) The effect on oncogenic transformation in NIH 3T3 cells correlates with the modulation of total protein translation efficiency. The y axis shows relative amounts of incorporated [^{35}S]-methionine after pulse labeling and TCA precipitation. *$p < 0.05$, **$p < 0.01$, error bars represent SD of three independent replicates. Data are representative of three independent experiments.

mouse miR-10a is found in several tissues (Beuvink et al., 2007; Landgraf et al., 2007).

Microarray analyses of mRNAs bound by miR-10a in ES cells demonstrate that miR-10a associates with a select set of mRNAs involved in protein translation. Using a photo-induced crosslinking approach, we demonstrate that miR-10a interacts directly with the isolated target mRNAs. Covalent linkage of miR-10a to the target mRNA furthermore allowed us to map the binding site by using primer extension assays. Nucleotide-specific crosslinking using 4-thiouridine nucleotides has been widely used in studies of the splicing machinery (Kim and Abelson, 1996; Yu and Steitz, 1997), but this is to our knowledge the first time a site-specific physical interaction has been demonstrated between a miRNA and its cognate target mRNA.

While most miRNAs studied so far have been found to target the 3′UTR, we demonstrate here that miR-10a binds RP mRNAs at the 5′UTR downstream of the conserved 5′TOP motif known to regulate translation. miRNA regulation via 5′UTR binding is not unprecedented, as miR-122 was previously found to bind the 5′UTR of hepatitis C virus and modulate viral replication (Jopling et al., 2005). We find that the miR-10a-binding sites are conserved across mammalian species and that miR-10a binding and function does not entail complete base pairing to the seed region of the miRNA. We furthermore provide genetic evidence for the binding site via the introduction of mutations in the miR-10a-binding site and subsequent functional rescue via ectopic expression of a mutant miR-10a holding compensatory mutations. Importantly, we find the ability to bind and regulate RP translation via the 5′UTR to be conserved in other members of the miR-10 family. Hence, this mode of regulation is likely not limited to tissues and cell types in which miR-10a is expressed.

Surprisingly, we find that miR-10a binding at the 5′UTR facilitates translational enhancement rather than repression. This is demonstrated at the level of endogenous RP mRNAs, using polysome profiling, and at endogenous RP synthesis level, where overexpressing miR-10a enhances RP protein synthesis and ribosome biogenesis. Blocking endogenous miR-10a furthermore results in reduced production of RPs. We recapitulate the enhancing effect of miR-10a on translation in reporter assays, where a 29 nt region of the *Rps16* 5′UTR confers sensitivity

(D–F) Interaction of the pS16-WT and pS16-comp 5′UTRs with miR-10a and the pS16-comp 5′UTR with miR-10a comp. Substituted nucleotides are indicated with arrows and are shown in red.

(G–I) Luciferase reporter assays with (F) pS16-WT, (G) pS16-comp, and (H) pS16-CM5 cotransfected with a *Renilla reniformis* luciferase control and the indicated miRNAs. Y axes show Firefly luciferase ratio to the *Renilla reniformis* luciferase control. *$p < 0.05$, **$p < 0.01$, error bars represent SD of three replicates. Data are representatives of at least four independent experiments.

(J) Affinity purification of the pS16-WT and pS16-CM5 vectors with biotinylated miR-10a. The y axis shows Firefly *luciferase* relative to a *Ubiquitin* specificity control as determined by RT-qPCR and is normalized to miR-10a affinity purification of pS16-WT. ***$p < 0.001$, error bars show SD of three experiments, and data are representative of three independent experiments.

to miR-10a modulation onto a *luciferase* reporter. The fact that miRNAs may also mediate enhancement of translation implies that these important regulators have more functions than hitherto expected. That these findings may be of more general significance is supported by recent data demonstrating that AGO2-containing complexes are involved in translational activation during serum starvation (Vasudevan and Steitz, 2007) and that some miRNAs can induce translation of target mRNAs during cell-cycle arrest (Vasudevan et al., 2007). It is important to note that we find miR-10a capable of both translational repression via interaction with binding sites in the 3′UTR and translational enhancement via binding to the 5′UTR of different groups of mRNAs. The same miRNA may therefore exert different functions dependent on the site of interaction.

miR-10a Is Functionally Connected to the 5′TOP Motif

Many of the mRNAs isolated with miR-10a contain a 5′TOP motif. In contrast to most capped mRNAs, which normally start with an A residue, 5′TOP mRNAs begin with a C residue followed by a stretch of 4–14 uninterrupted pyrimidines (Meyuhas, 2000). The significance of the 5′TOP in translation is well-established, and the 5′TOP motif is predominantly found in mRNAs involved in translation and ribosome biogenesis and confers sensitivity to mitogens and nutrients (Meyuhas, 2000). The PI3K/mTOR pathways have been shown to impinge on 5′TOP regulation, but the upstream signaling pathways are still largely unresolved and may differ between cell types (Tang et al., 2001). Several RNA-binding proteins have been shown to recognize and to some degree modulate the expression of 5′TOP mRNAs; however, the exact mechanism for 5′TOP function is still elusive (Crosio et al., 2000; Pellizzoni et al., 1998; Zhu et al., 2001). Several of our experiments point to a connection between 5′TOP regulation and miR-10a. We demonstrate that miR-10a can alleviate the translational repression induced upon 5′TOP mRNAs following amino acid starvation. The interplay between these regulatory factors suggests a role for miR-10a as a regulator of cellular stress responses. In support of this, we show a requirement for miR-10a in anisomycin-induced translation of 5′TOP mRNAs and that inhibition of miR-10a can decrease the induction of 5′TOP translation imposed by activated RAS-V12. We furthermore show that mutations in the 5′TOP motif render a reporter insensitive to the translation enhancement effect of miR-10a. The observation that the enhancing effect of miR-10a is sensitive to rapamycin suggests a requirement for signaling through mTOR. In conclusion, our data show an important function for miR-10a in 5′TOP regulation. We speculate in a mechanism in which miR-10a competes with a negatively acting factor binding downstream from the 5′TOP motif. The presence of such a negative regulator has been suggested in 5′TOP regulation (Biberman and Meyuhas, 1999), but the identity of an inhibitory factor in 5′TOP translational control has yet to be established.

miR-10a Stimulates Global Protein Synthesis and Promotes Cellular Transformation

Several studies have pointed to an important role of translational control in cancer development, and both of the major tumor-suppressors p53 and RB negatively regulate ribosome biosynthesis (Ruggero and Pandolfi, 2003). RPs have been found deregulated in many cancers (Bassoe et al., 1998; Ferrari et al., 1990; Kondoh et al., 2001; Zhang et al., 1997), and causative roles in cellular transformation have been demonstrated for several proteins involved in translation, among these RPS3a (Naora et al., 1998) and eIF4E (Lazaris-Karatzas et al., 1990). Furthermore, prominent oncogenes, such as MYC, have been shown to positively affect global protein production. Both MYC and NMYC directly bind and transcriptionally activate a cohort of genes encompassing both rRNA genes, RP genes, and other translational regulators (Boon et al., 2001; Coller et al., 2000; Grandori et al., 2005). We find that miR-10a mediates enhanced RP synthesis, with the functional consequence that global protein production is increased by 30% as measured by [^{35}S]-labeling of newly synthesized proteins. Similar findings have previously been published for MYC overexpression in mouse B cells (Iritani and Eisenman, 1999). Importantly, inhibition of endogenous miR-10a results in a 40% drop in protein synthesis, demonstrating the specificity and physiological relevance of the experiments and pointing to a central role for miR-10a in regulating the translational machinery via modulating the translation of RP and other 5′TOP mRNAs.

We speculated that miR-10a, via its ability to enhance global protein synthesis, would affect processes of cellular transformation. In agreement with this notion, RAS-V12-transformed NIH 3T3 cells formed more colonies in soft agar when transfected with miR-10a and fewer colonies when the endogenous miR-10a was inhibited, relative to control transfections. This modulation in transformation capacity was mirrored by the global protein synthesis capability of the cells. Interestingly, miR-10b (differing from miR-10a at a single nucleotide position) has been found overexpressed in glioblastomas (Ciafre et al., 2005) and was recently reported to play an important role in breast cancer metastasis via regulation of *HOXD10* (Ma et al., 2007). As none of the cell types employed in our experiments express noticeable amounts of *HOXD10*, this mechanism cannot account for the observed effects of miR-10a. We propose that members of the miR-10 family may facilitate tumorigenic processes via boosting the protein synthesis apparatus, although we cannot rule out additional pro-oncogenic functions for miR-10 members. In addition, miR-10a may contribute significant regulation and tissue-specific control of protein translation in general.

The identification of a miRNA that enhances translation of a specific subset of mRNAs further broadens the repertoire of small RNA functions. That binding occurs at the 5′UTR and likely in concert with recognition of another motif by a collaborating complex adds complexity to the mechanisms involved in miRNA action.

EXPERIMENTAL PROCEDURES

Cell Culture

Mouse E14 ES cells were cultured in complete Glasgow's modified eagle's medium (GMEM) supplemented with glutamine, nonessential amino acids, sodium puryvate, 10% ES cell-certified serum (all from Invitrogen), β-mercapto ethanol (Sigma-Aldrich), and 1 U/μl ES-GRO (Chemicon). The cells were grown on plates coated with 0.1% gelatine in PBS at 37°C and 6% CO_2. Cells were passaged every second day and medium renewed daily. HEK293 and NIH 3T3/RASV12 cells were maintained in complete Dulbecco's modified eagle's medium (DMEM) supplemented with 10% FCS (Biochrom) and penicillin/streptomycin

(Invitrogen). For soft agar assays, cells were seeded in 0.4% agarose type VII (Sigma) for determination of anchorage-independent growth in soft agar. Colonies were quantified 2–3 weeks later in the microscope.

Vector Construction and Reporter Assays

For reporter assays, HEK293 or NIH 3T3 cells were transfected in 96-well plates with 30 nM miRNA duplex or miRNA hairpin precursors (Ambion), 0.15 μg luciferase vector, and 0.02 μg Renilla vector (pRL-TK) using Lipofectamine 2000. Forty-eight hours after transfection, luciferase activity was measured using the Dual-Glo Luciferase Assay (Promega).

A pS16-hGH1 vector was kindly provided by Oded Meyuhas (Biberman and Meyuhas, 1999). The pS16-WT luciferase vector was constructed by PCR amplification of the promoter and the 5′TOP from pS16-hGH1 and inserting this product into the pGL3 enhancer vector (Promega) using KpnI and HindIII. Primer sequences are in Table S3. The pS16-CM5 and pS16-comp luciferase vectors were generated from the pS16-WT luciferase vector by using QuikChange Site-Directed Mutagenesis Kit according to the manufacturer's recommendations (Stratagene).

The miR-10a complementary vector was constructed by inserting an oligo containing two consecutive perfect matches to miR-10a 3′ to the luciferase gene in the pGL3 vector. Primer sequences are in Table S3.

Pull-Out Experiments

Affinity purification of miRNA targets were done as described previously (Orom and Lund, 2007) and RNA amplified and analyzed on microarrays Mouse 430 2.0 from Affymetrix according to the Affymetrix protocol. Microarray data were analyzed by normalizing each probe set signal to the total signal of the array and sorting according to the average probe set signal for the miR-10a experiments. Data have been deposited at ArrayExpress (Accession MEXP-1375). For the Ago2 western blot analysis, beads were boiled in Laemmli sample buffer and proteins separated on a 10% polyacrylamide gel. Primary antibodies to Ago2 were used 1:500 and were kindly provided by Ramin Shiekhattar (Chendrimada et al., 2005).

For the crosslinking experiments, cells were transfected with the 4-thiouridine miR-10a duplexes (30 nM). Two days after transfection, cells were lysed in pull-out lysis buffer and the cleared lysate irradiated on ice in small drops with 365 nm UV light for 10 min using an UVGL58 (Upland) apparatus at 3 cm distance. Total RNA was isolated by TRIzol (Invitrogen) extraction according to the manufacturer's recommendations. Covalently associated miRNA:mRNA complexes were purified using streptavidin beads as described (Orom and Lund, 2007). Sequences of primers used for quantitative RT-PCR are listed in Table S2.

Immunoprecipitation of Ribosomal Proteins

Cells were labeled in complete DMEM with 10% FCS for 1 hr with [^{35}S]-methionine added (20 μCi/ml final concentration) prior to lysis in RIPA buffer (0.15 M NaCl, 1% Igepal, 0.5% sodium deoxycholate [All from Sigma-Aldrich], 0.1% SDS, 0.05 M Tris-HCl [pH 8], 2 mM EDTA [Calbiochem], 1× Pefabloc [Roche]) and preclearing with protein A beads (Pharmacia). For each IP, 1.5 mg protein and 2 μg antibody were used and incubated at 4°C O/N. Protein A beads were added for 4 hr, isolated, and washed three times in RIPA buffer and proteins extracted by boiling in Laemmli sample buffer. The amounts of immunoprecipitated RPs were determined by using western blotting and incorporated radioactivity by exposure to a phosphoimager screen. Antibodies used were Rps6 and c-Myc (Cell Signaling), Rps16 (Abcam), and Rpl9 (Santa Cruz).

Protein Translation Assay and Ribosome Biogenesis

E14 cells were maintained in complete GMEM and transfected the day prior to assaying. For protein translation assay, cells were pretreated with 20 nM rapamycin or left untreated for 3 hr. Then [^{35}S]-methionine was added (20 μCi/ml final concentration) to the medium and incubated 15 min at 37°C and 6% CO2. Proteins were precipitated with 5% trichloro acetic acid, washed with PBS, released with 0.5 M NaOH and 0.5% SDS, and measured using a scintillation counter. For ribosome biogenesis assay, cells were labeled for 1 hr with ^{32}Phosphate (Perkin Elmer), total RNA extracted using TRIzol, separated on a 1.2% agarose formaldehyde gel, and transferred to a nylon membrane for PI exposure and staining with methylene blue.

Statistical Testing

All p values are calculated as one-tailed Student's t test with n = 3, unless otherwise stated.

Quantitative RT-PCR

Quantitative RT-PCR was done using the SYBR Green master mix and Real-Time PCR System 7300 from Applied Biosystems. miRNA quantitative RT-PCR was done using the TaqMan MicroRNA Assay from Applied Biosystems.

ACCESSION NUMBERS

Data have been deposited at ArrayExpress under accession number MEXP-1375.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, five figures, and four tables and can be found with this article online at http://www.molecule.org/cgi/content/full/30/4/460/DC1/.

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